Pressure Effects on Cells

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Introduction

Animals exhibit a variety of behavioural and physiological changes as a result of exposure to both constant raised pressure and to changes in pressure. Furthermore, having manifested changes at pressure animals frequently show some adaptation, either in terms of the frequency of signs of change or in the magnitude of the changes. In general, during compression above their normal ambient pressure both aquatic and terrestrial animals show increased locomotion, reduced co-ordination, impaired reflexes, tremor, seizures, paralysis, cardiac arrhythmia's, respiratory difficulty and ultimately death. Physiological changes that can be measured in the laboratory include changes in metabolism and thermo-regulation. In man more subtle physiological signs and symptoms are observed including nausea, sleep disruption, cognitive deficits and psychological responses (possibly related to stress). The changes in motor function and nervous system excitability apparently arise as a result of effects of pressure on the central nervous system (CNS) and have been termed collectively the High Pressure Neurological Syndrome (HPNS). This terms covers a complex set of neurological pathologies and although a single cause of HPNS is unlikely, if the major perturbations in CNS function are identified, then successful amelioration might provide substantial relief from HPNS.

The greatest pressures found in nature exist in the deep ocean; with the Mariana Trench in the Pacific Ocean 10.9 km deep, giving a bottom pressure of 109 MPa. Bacteria and a few animals have been discovered living at this pressure (Bruun, 1957). Although there are interesting biochemical and chemical changes at greater pressures (*e.g.* enzyme inhibition and protein coagulation at pressures above 100 MPa) these pressure are incompatible with life. Accordingly only pressures in the range from atmospheric pressure to approximately 100 MPa will be considered.

In air-breathing animals raised partial pressure of the respirable gases lead to; pulmonary oxygen toxicity, with prolonged exposure to oxygen partial pressures above 20 kPa, central nervous system oxygen toxicology, acute exposure to partial pressures of oxygen above 200 kPa, (Clark, 1982), nitrogen narcosis, exposure to nitrogen at partial pressures above 500 kPa, (Bennett, 1982) and elevated partial pressures of carbon dioxide lead to effects on respiratory responses, narcosis and hypoxia (Schaefer, 1969). However, in 1967 it was shown that helium acted essentially as a pure pressure transmitting fluid (Miller *et al.*, 1967) and this opened the way for analytic experiments on the effects of pressure on mammals, including man.

Manifestations of Pressure Effects in Cells

There are many elements within cells that may exhibit sensitivity to changes in ambient pressure including the cell membrane, ion channels located in the plasma membrane (which mediate cellular excitability amongst other functions), neurotransmitter receptors (which mediate both fast and slow neurotransmission), ion transporters and pumps (which also mediate cellular excitability and also electrical homeostasis) and the cellular cytoskeleton (which has important functions in intracellular

transport of materials and the regulation of cellular activity). These elements demonstrate differential sensitivity to pressure but the collective expression of these effects defines how various cell types will respond to pressure and hence how the organism as a whole will respond.

(a) Phospholipids, membranes

Phosphatidylcholines are the major membrane component in eukaryotic organisms and vital components of human lung surfactant and serum lipids. Accordingly, their phase-transition behaviour is of acute interest because of the impact that might have on a wide spectrum of biological processes, reviewed recently by Koynova & Caffrey (1998). The phase transition temperature (gel to liquid-crystalline) may be altered and the bilayer may be compressed by pressure. These changes may, in turn, affect the function of cellular proteins crucial in controlling the excitability of cells and the synaptic communication between cells.

Liu & Kay (1977) reported that the isothermal compressibility of dipalmitoyl-phosphatidylcholine (DPPC) bilayers in pure water and above the transition temperature was about 1 $cm^3.mol^{-1}.MPa^{-1}$. Compression is anisotropic with increasing pressure a hydrated DPPC bilayer above its transition temperature increased in thickness by 0.07%.MPa^{-1}. However, a net volume reduction occurred because there was a simultaneous reduction in lateral spacing (Stamatoff *et al*., 1978).

The effect of pressure on the phase-transition temperatures is well documented for a variety of phosphatidylcholines (Koynova & Caffrey, 1998). The rippled gel-phase to liquid crystalline transition temperature for symmetrical fully-hydrated phosphatidylcholines of chain-length 14 increases from 27°C at 15 MPa to 50°C at 110 MPa. The sensitivity of the temperature of the chain melting transition to pressure has been found to remain constant, at approximately 0.24^{o}C.MPa^{-1}, for a variety of saturated and unsaturated phosphatidylcholines (Winter & Pilgrim, 1989). At high pressures, above 100 MPa, additional gel phases have been reported (Prasad *et al*., 1987). These may be of relevance to organisms adapted to living at very great depths.

In the range of pressures that have a profound effect on neurological function, 2.5 MPa to 10 MPa, the transition temperature would change by less than 2°C. This is borne out by experiments on human erthyrocyte membranes equilibrated with spin-labelled stearic acid in which little effect on bilayer fluidity was observed at 10 MPa (Finch & Kiesow, 1979). However, it is possible that a specialised region of the lipid bilayer has a transition temperature close to normal body temperature. In this case, if the transition occurs over a narrow temperature range, say 1°C, then a relatively small increase in pressure might profoundly reduce membrane fluidity.

The role of phospholipids in cell signalling must also be considered, where the association with the basal membrane, either the plasma membrane or that of an intracellular organelle, may indeed be critical and capable of being influenced by pressure changes below 10 MPa. The inositol phospholipids are a family of membrane lipids found in all mammalian and plant cells that have important roles in cellular functions. They covalently anchor many cell surface proteins (Low, 1987) and they are a source of second messengers that link metabotropic receptor activation with a cellular function (Higgins, Hitchin & Low, 1989). A study of an inositol phospholipid from soya bean in a bilayer environment examined the effects of pressure on intra- and inter-molecular interactions (Carrier & Wong, 1996). In hydrated inositol phospholipid bilayers at normal temperature and pressure the phosphate groups and a major portion of the carbonyl functions undergo strong hydrogen bonding with water molecules. Increasing pressure, up to 600 MPa strengthens these bonds. Above this pressure, water is forced from the hydrophobic interior but the phosphate head group remains strongly hydrogen bonded. In dehydrated inositol phospholipid

bilayers increasing pressure up to 200 MPa causes a conformational change that results in a smaller area per molecule and an extension of the inositol moiety away from the phosphate group. These changes may be of greater significance for physiological function, especially if they occur to any marked degree at lower pressures.

(b) Cellular Proteins

Hydrostatic pressure effects on proteins in aqueous solution have been extensively studied (Heremans, 1982; Sibenaller & Somero, 1989; Somero, 1990). In general, water soluble proteins (and polypeptides) may undergo limited self-conformational changes when exposed to pressure. These processes involve small negative volume changes (ΔV) and include transitions in the spatial coil structure (-1 to -3 $cm^3.mole^{-1}$), reversible denaturation (-20 to -75 $cm^3.mole^{-1}$) and some interactions with small molecules (-1 to -10 cm^3 $mole^{-1}$). Other self-organization processes, such as isomerization, exhibit positive ΔV (30-50 $cm^3.mole^{-1}$). Interactions between larger molecules are associated with larger positive volume changes; these include protein-protein association (25-390 $cm^3.mole^{-1}$), self-assembly of polymeric proteins (60-140 $cm^3.mole^{-1}$) and binding of cofactors or substrate molecules (30-70 $cm^3.mole^{-1}$). The volume change of these interactions depends on several, sometimes opposing, mechanisms such as the degree of ligand ionization, of protonation of protein groups and hydration of the protein surface.

Enzymes

Enzyme catalysed reactions often involve multiple sub-reactions and it can be difficult to pinpoint the critical, rate-determining step. With high substrate concentration, the rate determining step has been shown to be the dissociation of the enzyme-substrate complex to yield the reaction products (Laidler, 1951). In this case the effect of pressure will be determined by the volume change associated with this process. For a variety of enzymes (including myosin ATPase, trypsin, sucrase, myokinase) this volume change has been shown to be negative and of the order -10 $cm^3.mole^{-1}$ to -40 $cm^3.mole^{-1}$ (Macdonald, 1982). Thus pressures greater than 40 MPa would be required to significantly increase the rate of reaction. Another group of enzymes (including creatine kinase, alkaline phosphatase and a number of ATPase's) have been shown to have a positive volume change on dissociation of enzyme and substrate (Macdonald, 1982). Pressure would therefore inhibit the action of these enzymes but again a significant effect is only observed at very great pressure. However, in physiological conditions the concentration of the substrate is not high when compared to the enzyme concentration. Under these conditions association of enzyme with substrate or enzyme-substrate dissociation may be rate determining. Morild (1977) has given an example of the detailed analysis required under these conditions for alcohol dehydrogenase.

Although very high pressures are generally required to modify the activity of an enzyme in isolated conditions (Balny *et al*., 1997) much lower pressures (<10 MPa) can modify enzyme activity by altering the membrane environment of the enzyme (Sébert, 1997). Relatively low pressure (10 MPa) has been shown to modify glycolysis in Chinese freshwater crabs, increasing flux per unit time more than threefold (Sébert *et al*., 2000). Experiments on the inactivation of creatine kinase by high pressure indicate that the low pressure inactivation arises from a change in environment which precedes the dissociation of the enzyme dimer and the unfolding of the hydrophobic core, at pressures above 300 MPa (Zhou *et al*., 2000).

It appears that under physiological conditions enzyme catalysed reactions are not altered by pressure acting directly on the enzyme itself (until extreme pressures are reached that are incompatible with biological processes) but rather arise through a modification to the environment

of the enzyme. The action of pressure on enzymatic processes must therefore be studied under physiological conditions.

Voltage-gated ion channels

Voltage-gated channels are proteins produced by a super-family of related genes and thus exhibit remarkable homology in their structure and similarity in basic functions. They are usually activated by membrane depolarization and deactivate when the membrane potential returns to its resting level. Many of the channels also exhibit various degrees of voltage-dependent channel inactivation, *i.e*. channel closing during maintained membrane depolarisation. The conduction of electrical impulses (action potentials) along neurons, axons, and muscle fibres is based on a delicate interplay between voltage-activated Na^+ and K^+ channels. Upon threshold depolarisation from the resting potential, Na^+ channels open rapidly to cause fast depolarisation this is followed by slower channel inactivation. In parallel, K^+ channels, opening at a slower rate, re-polarise the membrane. It is this voltage and time dependent channel interplay that accounts for the transient voltage "spike" of the action potential.

Bruner and Hall (1983) made the first attempt to understand pressure effects on a membrane-bound polypeptide channel, the pore-forming antibiotic alamethicin. When incorporated into artificial lipid bilayer membranes, these peptides form ion-conducting oligomers composed of a variable number of units (about 10) in a voltage-dependent fashion. When exposed to helium pressure up to 100 MPa, both the voltage-dependent "opening" and the "closing" reactions became slower, indicating ΔV^* on the order of 100 $cm^3.mole^{-1}$ in both directions. Kinetic considerations at pressure also suggested that the alamethicin channel has at least two closed states which are indistinguishable at normobaric pressure. Neither the voltage-dependence of channel opening nor the single-channel conductance was affected by pressure. These results imply that the formation and disassembly of a functional alamethicin channel are associated with molecular rearrangements that cause transient increases in free volume of the entire channel. In addition, the flow of ions through the channel does not require significant volume changes, very similar to free-ion diffusion in aqueous solutions. Although the alamethicin ‘pore’ is not a biological ion channel, many of these pressure effects were later observed for membrane bound ion channels.

Na^+ channels

Early studies on Na^+ channel currents were carried out in squid giant axons (Henderson & Gilbert, 1975; Shrivastav *et al*. 1981; Conti *et al*. 1982a,). Pressure (up to 60 MPa) slightly depresses Na^+ current amplitude and slows the kinetics of activation and inactivation of the current. Inactivation seems more affected than the activation, but only a slight change in the voltage dependence of the steady-state inactivation is observed. These data are consistent with ΔV^* in the range 30-70 $cm^3.mole^{-1}$. Conti *et al*. (1984) and Heinemann *et al*. (1987a) also observed a considerable ΔV^* associated with the channel gating current (17-28 $cm^3.mole^{-1}$). Gating current precedes channel opening. It represents the movement of charge as a result of conformational changes in the channel voltage sensor, rather than the flow of ions through the channel. Thus, about 50% of ΔV^* for the channel opening is attributed to the gating currents.

Similar responses were observed using other invertebrate preparations, such as snail (*Helix*) neurons (Harper *et al*., 1981) and lobster (*Panulirus*) axons (Grossman & Kendig, 1984). However, inactivation of the current in *Helix* was not affected by pressure. The lobster axons revealed a transient increase of the current upon fast pressurization to 5-10 MPa, which reversed to leave a moderate decrease after 10-15 min of exposure. The findings in vertebrate cells are more diverse. In

single isolated myelinated axons of frogs (*Xenopus*) (Kendig, 1984) similar pressures considerably slow the rate of development of inactivation so that the channels stay open longer and more channels are available for opening. In another frog (*Rana*), action potential Na^+ currents recorded under the perineurial sheath of peripheral motor nerves (Grossman *et al*., 1991b) were reduced by 25% at 10 MPa and the kinetics were slightly slower. Recent studies of Na^+ action potential currents in mammalian neurons revealed only a 10% decrease in amplitude and rise time at 10 MPa in single cerebellar Purkinje cells (Etzion & Grossman, 1999) and hippocampal pyramidal cells (Southan & Wann, 1996). A study on non-neuronal bovine adrenal chromaffin cells (Heinemann *et al*., 1987a) revealed similar pressure effects with considerable slowing of inactivation. Detailed statistical analysis of the steady-state current fluctuations in these cells (Heinemann & Conti, 1992) indicated that the single channel current amplitude was not affected, even at 45 MPa hydrostatic pressure, whereas the number of functional channels was reduced, by up to 50%.

In summary, in spite of species differences, membrane bound neuronal Na^+ channels seem quite resistant to compression. Pressure only slightly reduces the mean current amplitude by a minor slowing of the activation process or by decreasing the number of functional channels. In some neurons in which the inactivation is impeded more than the activation process, pressure may enhance or elongate, sometimes transiently, the Na^+ current.

K^+ channels

Voltage clamp studies of the non-inactivating "delayed rectifier" K^+ current in invertebrate squid (Henderson & Gilbert, 1975; Shrivastav *et al*., 1981; Conti *et al*., 1982b), snail (Harper *et al*., 1981) and lobster (Grossman & Kendig, 1984) axons reported slowing of the rising phase of the current after step depolarization, which corresponds to a ΔV^* of 25-60 $cm^3.mole^{-1}$. However, the steady-state plateau current was increased. In the lobster (reminiscent of the Na^+ current response) this increase developed with time after an initial decrease in the current. Similar K^+ current augmentation was postulated for mammalian cells. Analysis of the firing pattern of Ca^{2+} action potentials in guinea pig Purkinje cells indicated pressure enhancement of this current (Etzion & Grossman, 1999). Increased K^+ current was also proposed as the basis for slowing of the sinus-node pacemaker activity in various mammalian hearts, as a result of slower diastolic depolarization (Ornhagen & Hogan, 1977). Another potassium channel type, whose current is inactivating, was studied in snail (I_{AK}) (Harper *et al.*, 1981) and recently in mouse *Shaker* B K^+ channels and mutants thereof, expressed in *Xenopus* oocytes (Schmalwasser *et al*., 1998; Meyer & Heinemann, 1997). Activation of both currents is depressed by pressure (ΔV^*= 45 $cm^3.mole^{-1}$). However, whereas inactivation in the snail was not affected by pressure, the two different types of inactivation in the mouse channels were differentially affected. While rapid N-type inactivation, mediated by the so-called "ball-and-chain" mechanism is slowed down at high pressure (ΔV^* = 22-54 $cm^3.mole^{-1}$), the slow C/P-type inactivation is accelerated (ΔV^* = -58-125 $cm^3.mole^{-1}$).

A different type of K^+ channel, which are activated by increased intracellular Ca^{2+} ($[Ca^{2+}]_i$) rather than a change of membrane potential, are also modulated by pressure. Pressure exposure probably decreases this channel conductance in rat hippocampal pyramidal neurons, as evident from the change in their action potential after-hyperpolarizing potential (AHP) and the increase in firing frequency (Southan & Wann, 1996). Very similar results were recently obtained for pressure sensitive neurons in rat brain stem (solitary complex) compressed by helium to only 0.4 MPa (Dean & Mulkey, 2000). In contrast, high pressure increased the probability of openings of Ca^{2+}-activated BK-type channel in bovine chromaffin cells (Macdonald, 1997).

In summary, pressure slows the kinetics of activation and inactivation of membrane bound

potassium channels as reported above for sodium channels. However, due to the larger impact on inactivation rather than activation, and the existence of more than one molecular mechanism of inactivation, which may respond differently to pressure, pressure, overall, quite often increases the channel current

Ca^{2+} channels

Many fundamental cellular processes are controlled, modulated or triggered by cytosolic Ca^{2+} levels. Muscle contraction, ciliary movement, synaptic transmission, egg fertilization and activation of various second messenger systems are just a few of these processes. Several mechanisms such as membrane pumps, ion exchangers and buffering systems rigorously keep the resting cytosolic $[Ca^{2+}]$ as low as 0.1 μM. A transient increase of cytosolic $[Ca^{2+}]$ may serve as the signal for the targeted process at the relevant cellular compartment. Ca^{2+} may enter the cell from the external fluid via various types of channels or be released from internal stores via specific receptor channels.

The data on Ca^{2+} channel function at pressure are limited. Measurements of radiolabelled Ca^{2+} influx into synaptosomes (sealed vesicles from broken nerve terminals, containing Ca^{2+} channels and the synaptic release apparatus) revealed that pressure depresses the depolarization-dependent Ca^{2+} influx (Gilman *et al.,* 1986b). It is of interest to note that, in the same preparation, pressure slightly increased Ca^{2+} influx through an artificially added Ca^{2+} ionophore (A23187) (Gilman *et al*., 1991). Otter and Salmon (1985) have provided indirect evidence for the effect of hydrostatic pressure on unclassified calcium channels in *Paramecium.* The brief reversal of swimming direction, which is Ca^{2+} influx-dependent, that normally occurred when the protozoan encountered the wall of the pressure chamber was inhibited by 10 MPa hydrostatic pressure. However, they failed to demonstrate an inhibition of Ca^{2+} influx in a later study in mouse fibroblasts (Crenshaw & Salmon 1996).

Several high voltage-activated Ca^{2+} channels have been described and termed N-, L-, P-, and Q-type, based on their kinetics, voltage range of activation and inactivation and pharmacological properties. An additional channel, which is resistant to all known channel-blocking drugs, was termed R-type. A low voltage-activated channel, which has been less well studied, is termed T-type. It has been confirmed, using molecular biological techniques, that these channels also differ in their α_1 pore-containing protein subunit. Various Ca^{2+} channels mediate synaptic transmission between CNS neurons in the hippocampus (Takahashi & Momiyama, 1993; Luebke *et al*., 1993; Wu *et al.*, 1998) and cerebellum (Regehr & Mintz, 1994; Mintz *et al*., 1995). Specific GABA containing inhibitory interneurons in the hippocampus also utilize different Ca^{2+} channels for their release processes (Poncer *et al*. 1997). Furthermore, colocalization of different Ca^{2+} channels in a single motor nerve terminal of frog (Mallart, 1984; 1985), mouse (Penner & Dreyer, 1986) and CNS terminals (Lemos & Nowycky, 1989) has also been shown.

The effect of pressure on two types of colocalized Ca^{2+} currents was first tested in the frog motor nerve (Grossman *et al.* 1991b; Kendig *et al*., 1993). In addition to the action potential Na^+ current of the axons, blocking K^+ channels using TEA, revealed a slower Ca^{2+}-dependent current comprised of fast (I_{CaF}) and slow (I_{CaS}) components (Mallart, 1984) that reflect inward Ca^{2+} current at the terminals. Pharmacological analysis indicated that I_{CaF} is carried by N-type channels and I_{CaS} by L-type channels. Helium pressure (6.9 MPa) suppressed N-type channels by about 87%, whereas L-type channels were much less sensitive to pressure. Part of the effect could be antagonized by increased $[Ca^{2+}]_o$. Similar results were obtained when the terminal was depolarized directly instead of by natural nerve stimulation. These data suggest that pressure may exert a differential effect on various types of Ca^{2+} channels at nerve terminals. In fact, Heinemann *et al*. (1987a) have reported

that L-type Ca^{2+} current was not sensitive to high pressure in mammalian chromaffin cells. Recently it has also been reported that similar results are obtained for P-type Ca^{2+} action potentials in guinea pig cerebellar Purkinje cells (Etzion & Grossman, 1999). Finally, Etzion and Grossman (2000) have suggested that pressure blocks mainly the N-type (and much less so other types) Ca^{2+} channel-dependent component of the synaptic response between the parallel fibers and Purkinje cells' synapse in guinea pig cerebellum.

Given the large diversity even within this single channel 'family' and the limited data available, pressure effects can not be generalised. A systematic study will be necessary in order to analyse the pressure response of the various Ca^{2+} channels. It is conceivable that synapses utilising N-type Ca^{2+} channels will be more vulnerable to pressure exposure than others. The extent of the consequent behavioural modification will be dependent on the role of such a synapse is playing in the network activity.

Receptors

Receptors are proteins that bind affector substances and transduce that binding into a physiological effect. They are grouped, on the basis of structure and signal transduction mechanism, into 4 superfamilies; ionotropic, metabotropic, ligand-gated enzymes and protein-regulating receptors. Ionotropic receptors, also known as ligand-gated ion channels, are responsible for regulating fast synaptic neurotransmission. Activation of the receptor by binding a neurotransmitter causes a conformational change that allows ions to pass through a channel integral to the protein. Metabotropic receptors, also known as G-protein coupled receptors, bind neurotransmitters, hormones and neuropeptides that cause a conformational change in the receptor. In turn, this allows the receptor to bind to and activate a G-protein (guanyl nucleotide-binding protein). The activated G-protein then activates another protein or enzyme or ion channel, the "so-called" second messenger, which effects the cellular response. These receptors primarily mediate slower processes than the ionotropic receptors. This is by far the largest family of receptors with approximately 1% of the human genome encoding the different proteins. The ligand-regulated enzymes (*e.g.* insulin receptor) are a smaller family of receptors responsible for a variety of homeostatic systems. Lastly, the protein synthesis-regulating receptors are found in the cytosol and the nuclear membrane and, in response to binding hormones, steroids or retinoic acid, affect the transcription of DNA to RNA and hence the synthesis of proteins.

Ionotropic Receptors

The ionotropic receptor superfamily is composed of three families, categorised on the basis of amino acid homology in the primary structure; the Cys-loop receptors, the glutamate receptors and the P2X receptors. The Cys-loop family comprises the nicotinic acetylcholine receptor (nACh), the γ-aminobutyric acid-A receptor ($GABA_A$), the glycine receptor (Gly) and the third member of the 5-hydroxytryptamine family of receptors ($5\text{-}HT_3$). The nACh and $5\text{-}HT_3$ receptors both gate a cation channel (permeable to Na^+, K^+ and Ca^{2+}) and are consequently responsible for mediating excitatory synaptic transmission. The $GABA_A$ and Gly receptors both gate a Cl^- channel and are thus responsible for mediating inhibition. The glutamate receptor family, activated by glutamate and aspartate, comprises three distinct receptors known by the agonists that selectively activate them, the N-methyl-D-aspartate sensitive receptor (Glu^{NMDA}), the α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid sensitive receptor (Glu^{AMPA}) and the kainic acid sensitive receptor (Glu^{KA}). These receptors all gate a cation channel, and are therefore excitatory, but Glu^{NMDA} is far more permeable to Ca^{2+} than the other two members of the family. Finally, the P2X familiy of receptors is activated by adenosine triphosphate (ATP). They gate a cation channel, therefore mediating fast

excitatory synaptic transmission. At present nothing is known about the pressure sensitivity of the P2X family of receptors.

Cys-loop Receptors

The nACh receptor exists in two major forms, the neuromuscular junction and neuronal types. The neuromuscular junction form of the receptor is probably the most extensively studied of all receptor types and much of our knowledge of receptor structure and function has been derived from studies on this receptor (see for reviews Galzi *et al*., 1991; Unwin, 1993; Ortells & Lunt, 1995). The effect of pressure on the neuromuscular form of the receptor has been investigated by Heinemann *et al*. (1987b). They calculated a volume of activation for the nACh receptor of some 48 $cm^3.mol^{-1}$ but found no evidence for any significant effect on volume of activation of the receptor by pressures less than 20 MPa. The neuromuscular form of the nACh receptor is a pentameric structure composed of $(\alpha)_2\beta\varepsilon\delta$ subunits. Acetylcholine binds at the interface between each α and one other subunit. The neuronal form of the receptor whilst still pentameric only comprises two subunits α and β but it has a far richer variety of isoforms since there are seven α and four β subunits, which combine in unknown stoichiometries. It is possible that the neuronal form of the receptor is sensitive to pressure even though the neuromuscular form is not.

The effect of pressure on the $GABA_A$ and glycine receptors has been investigated by isolating mammalian receptors using heterologous expression in *Xenopus* oocytes. The effects of pressure on the dose-response relationships for $GABA_A$ and glycine superfused onto oocytes micro-injected with mRNA extracted from rat brain and spinal cord have determined. Glycine receptors show no change in the maximum response but a significant increase in the EC_{50} (60%) at 10MPa (Shelton *et al*., 1993) whereas pressure has no effect on the $GABA_A$ receptor (Shelton *et al*., 1996). The effects of pressure on the glycine receptor become progressively greater at higher pressures. It was calculated that the volume change on activation of the glycine receptor was 110 $cm^3.mol^{-1}$, considerably greater than for the nACh receptor. This would explain why the glycine receptor is more sensitive to pressure than the nACh receptor. A recombinant $\alpha1$ human glycine receptor has been expressed in *Xenopus* oocytes and its pressure sensitivity established (Roberts *et al*., 1996). In this case the receptor was somewhat more sensitive to pressure, with a volume of activation of 149 $cm^3.mol^{-1}$. Since this homomeric receptor apparently binds at least three glycine molecules when activated, it lends support to the suggestion that the neuronal form of the nACh receptor may show more pressure sensitivity than the neuromuscular form.

A classical interpretation of the dose-response data obtained for the glycine receptor (both rat and human homomeric $\alpha1$) would be that pressure is acting as a simple competitive antagonist. This action could be manifest either as a reduction in the affinity of glycine binding or in the transduction of binding to channel opening. A direct effect on channel opening or lifetime seems unlikely since the closely related $GABA_A$ receptor is unaffected. The apparent volume change on activation was large for simple chemical equilibria and indicates that considerable conformational changes occur when glycine binds. The effect of pressure may therefore be envisaged as opposing the conformational change that follows glycine binding thereby reducing the effective inhibitory transmission available.

Glutamate Receptors

Glu^{NMDA} receptors expressed in *Xenopus* oocytes following injection of mRNA extracted from rat cerebellum show a marked potentiation (128%) at 10MPa (Williams *et al*., 1996; Daniels *et al*., 1998), implying that overall there is a negative ΔV associated with activation of this receptor. In

contrast, Glu^{KA} receptors, expressed in *Xenopus* oocytes following injection of mRNA extracted from rat whole brain, are relatively unaffected, showing little potentiation of the maximum response (<14%) and no change in the EC_{50} (Daniels *et al*., 1991; Shelton *et al*., 1993). However, the response of Glu^{NMDA} receptors is not straightforward. Glu^{NMDA} receptors are composed of two subunits, R1 and one of R2A, R2B, R2C and R2D, in unknown stoichiometry. All Glu^{NMDA} receptors contain at least one R1 subunit. The different R2 subunits control the conductance of the receptor, thus R1/R2A and R1/R2B are high conductance receptors (40-50 pS) whilst R1/R2C and R1/R2D are low conductance receptors (18-38 pS). These two groups of receptor also have different sensitivity to voltage-dependent blockade by Mg^{2+} and thus different activation properties. They are also found differentially expressed in the brain. R1 is uniformly distributed throughout the brain. R2A is widely distributed throughout the cerebellum and cortex, R2B is found in the cortex and R2C is largely confined to the cerebellum (Monyer *et al*., 1992). In *Xenopus* oocytes the R1 subunit will express functional receptors without co-expression of one of the R2 subunits, probably by functional combination with an endogenous protein subunit. These receptors are insensitive to pressure (Daniels, unpublished observations), suggesting that the pressure sensitivity observed with Glu^{NMDA} receptors expressed using a rat brain mRNA extract arose because one or more of the R2 subunits is pressure sensitive. Recombinant receptors R1/R2A, R1/R2B and R1/R2D expressed in *Xenopus* oocytes were not sensitive to pressure (Daniels, unpublished observations). To date the R1/R2C receptor has not been tested at pressure. However, given the localisation of this receptor to the cerebellum and the well-known association of cerebella function and motor control it would be surprising if this particular receptor type were not pressure sensitive. This would also be in accord with the initial observation that Glu^{NMDA} receptors from rat cerebellem were pressure sensitive.

The results with the Glu^{NMDA} receptor demonstrate the need to carefully analyse the pressure sensitivity of all the different receptor subtypes before reaching an overall conclusion regarding the pressure sensitivity of the ionotropic receptors. All the ionotropic receptors share a rich subunit composition. Thus, unless some common structural motif can be identified that conveys pressure sensitivity, there is a major challenge to define the pressure sensitivities of this class of receptor.

Metabotropic Receptors

The metabotropic family of receptors is the largest, with over 100 receptors now identified. They possess seven α helical transmembrane regions with a large intracellular loop joining transmembrane segments 5 and 6 and a long C-terminal tail from segment 7. The tail and loop contain binding sites for heterotrimeric guanine nucleotide binding proteins (G-proteins). Activation of G-proteins results in various biochemical and electrical effects that produce physiological effects. Three principal intracellular signalling pathways are activated by G-proteins. The first involves either the activation (Gs) or the inhibition (Gi) of adenylyl cyclase (AC). Stimulation of AC leads to the production of cyclic adenosine phosphate (cAMP) that in turn upregulates the formation of protein kinase A (PKA). The second pathway (Gq/G11) involves activation of phospholipase C (PLC), which leads to the formation of inositol 1,4,5-triphosphate (IP_3) the release of Ca^{2+} from intracellular stores and the activation of calcium-calmodulin-dependent protein kinases. Activation of PLC also leads to the formation of diacylglycerol (DAG), which activates protein kinase C (PKC). The third pathway (Go) involves the activation of phospholipase A_2 (PLA_2), which produces arachidonic acid (AA) that is the precursor for the formation of various metabolites such as prostaglandins, thromboxanes and leukotrienes. The formation of DAG can also lead to AA formation.

A number of receptors in an individual cell may activate a single G protein or one receptor may regulate more than one G protein. Additionally, one G protein can regulate several effectors (*i.e.*

AC, PLC, PLA_2). Metabotopic receptors, therefore, provide complex networks of convergent and divergent interactions that permit extraordinarily versatile regulation of cell function. Although many of the actions they mediate are slow, compared to those mediated by the ionotropic receptors, nevertheless the complexity of interactions invites speculation that pressure many modulate some of these molecular interactions. In doing so the excitability of neurons may be altered possibly contributing to the acute expression of high pressure nervous syndrome but perhaps more crucially regulating adaptive mechanisms to exposure to high pressure.

Pharmacological experiments have indicated that pressure may affect both dopamine (DA1, DA2) and serotonin ($5\text{-}HT_{1b}$, $5\text{-}HT_{2c}$) receptors (Abraini & Rostain 1991; Abraini & Fechtali, 1992; Kriem *et al.*, 1996; 1998). Dopamine D1 receptors couple through Gs proteins and hence activate AC, up-regulate cAMP and activate PKA. Dopamine D2 receptors couple through Gi/o proteins and can therefore inhibit AC and separately activate PLA_2 thus activating AA. Serotonin $5\text{-}HT_{1b}$ receptors also couple through Gi/o proteins. However $5\text{-}HT_{2C}$ receptors couple through Gq proteins that activate PLC leading to the release of intracellular calcium and PKC. These experiments, whilst indicating that the cellular processes mediated by these metabotropic receptors may be implicated in HPNS they cannot indicate whether pressure is modulating receptor function or steps in the intracellular signalling pathways activated by the receptors..

It has been found recently (Daniels, unpublished results) that the metabotropic receptor for the bacterial peptide N-formyl-L-methionyl-L-leucyl-L-phenylanaline, which couples through a Gq protein, when expressed in *Xenopus* oocytes is not sensitive to pressure. This suggests that none of the PLC pathway reactions result in a large volume change with which pressure could interact. In turn this suggests that it is likely that the modulation by pressure of the pharmacological response of $5\text{-}HT_{2C}$ receptors arises from a direct effect of pressure on the $5\text{-}HT_{2C}$ receptor and not on the intracellular pathway (PLC medicated) to which it couples. In separate experiments it was found that direct stimulation of a G protein endogenous to *Xenopus* oocytes by fluoride ions was potentiated by pressure (Daniels, unpublished results). Analysis indicated that the G protein stimulated by F^- was a member of the Gs family and therefore would positively couple to AC. Thus pressure appears to modulate either the upregulation of AC, the production of cAMP or PKA (or a combination of these interactions). Pressure may therefore decrease the inhibitory actions of D2 receptors and stimulate the excitatory actions of D1 receptors. Furthermore, since both D1 and D2 receptors are predominantly expressed in the striatum and are known to be involved in motor control (*cf* Parkinson's disease; Gibb, 1992; Fearnley and Lees, 1994) this result does suggest that dopamine receptors may in part mediate the expression of acute HPNS.

Transporters and pumps

Membrane transporters (*e.g.* Na/K pump, Na/K/2Cl cotransporter) have a crucial role in maintaining ionic and osmotic balance in a wide variety of cells. It is difficult to predict what effect pressure would have on these transport systems, just as it is difficult to predict the effect on enzyme catalysed reaction rates. In human red cells Na^+ and K^+ are transported across the membrane by the sodium pump, chloride-dependent sodium-potassium cotransport and by a residual passive leak. It has been shown that over the pressure range atmospheric to 40 MPa the sodium pump is progressively inhibited, passive diffusion is markedly potentiated and the cotransport is initially inhibited but little further affected by pressures over 10 MPa (Hall *et al*., 1982). The authors suggested that the initial formation of the activated-K^+-membrane pore complex involving loss of water from the K^+ hydration shell was the rate-determining step in the sodium pump that was sensitive to pressure. No explanation was offered for the inhibition of the Cl^--sensitive Na/K cotransporter. However, since the inhibition occurred during the initial pressurisation from

atmospheric to 10 MPa it would seem likely that this reflects a modification to the transporter protein-membrane environment and not a direct effect of pressure on the transporter protein itself. The increase in the passive flux by pressure has been ascribed to a pressure-dependent change in cell morphology (from biconcave disc to cup-shaped forms) leading to activation of a volume-sensitive KCl transport pathway (Hall & Ellory, 1986).

Glucose is transported across the red blood cell membrane by a transporter that alternatively presents binding sites to the exterior and interior of the cell (Thorne *et al*., 1992). The rates of re-orientation between inward- and outward-facing conformations are much higher for the glucose-bound protein than the unbound protein. Additionally, the rate constants for inward re-orientation exceed those for outward re-orientation. It was found that pressure specifically inhibits the translocation of glucose and not the binding of glucose to the carrier protein (Thorne *et al*., 1992).

Chondrocytes are the cells responsible for producing the extracellular matrix (cartilage) which protects joints. In part they must do this by sensing pressure changes and altering the composition of the matrix accordingly. It is possible that this is achieved by the effect of pressure on both specific and passive membrane transporters in these cells (Hall, 1999). In contrast to red blood cells, pressure on chondrocytes has been shown to inhibit the Na/K pump, the Na/K/2Cl cotransporter and the passive K^+ permeability (Hall, 1999). Increasing pressure therefore would reduce internal K^+ concentration ($[K^+]_i$) and increase $[Na^+]_i$ thereby inducing cell swelling. These changes are known to affect chondrocyte matrix synthesis.

In summary, alterations by pressure to transporter function are likely to be replicated across a wide spectrum of cell types and are probably not restricted simply to the transport of ionic species across membranes. Most neurotransmitters rely on specific transport systems to terminate their action at a synapse. Clearly inhibition of neurotransmitter re-uptake would have profound effects on neuronal function. As yet the effect of pressure on these systems has not been studied and it must be stressed that, as with enzyme function, the environment of the transporter may be critical to the manifestation of pressure effects.

(c) Cytoskeleton and motor proteins

Multisubunit or polymeric proteins are usually dissociated by high pressure, although the pressure sensitivity varies between different proteins and even, apparently, for the same protein in different species. Cytoskeletal proteins such as f-actin (‘thin’ stress fibers), tubulin (‘thick’ microtubules) and various ‘intermediate filaments’ stabilize the morphology of cells. Actin and tubulin also provide the structural basis for the action of motor proteins such as myosin, dynein and kinesin that generate muscle contraction, ciliary beating, and chromosome movement and intracellular transport, respectively.

In recent years a large number of new ‘structural’ proteins have been described that are involved in cell development and cellular plasticity. A number of these proteins are responsible for intercellular adhesion in general and at specific sites, such as neuromuscular or neuroneuronal synapses. Other proteins are engaged in binding transmembrane proteins (*e.g*. receptors or ion channels) to the membrane and may form clusters of these molecules. Many of these proteins contain, in addition, binding sites for specific enzymes (usually kinases or phosphatases) that are associated with the modulation of the activity of those receptors or channels. Unfortunately, there is no information available on the effects of pressure on these complexes of proteins.

Actin

Actin is involved in various types of cell locomotion. Associated with different other proteins, filaments of actin may be arranged in bundles (cell microvilli), as a 'gel forming' mesh (beneath cell membranes), in short fragmented sections and as a double strand of the 'thin filament' of the muscle fibre. By interacting with the 'thick filaments' of myosin they produce muscle contraction. Similar but not identical assembly is found in specific regions of non-muscle cells that are capable of contracting (*e.g*. desmosomes, stress fibres). Self-assembly (polymerisation) of the globular actin (g-actin) monomers to a single filament (f-actin) *in vitro* is associated with a large increase in volume ($\Delta V \sim$ 100-140 $cm^3.mole^{-1}$) for one atmosphere-adapted animals (see for review Crenshaw *et al*., 1996). Pressure then should promote dissociation of the filamentous protein since it involves negative volume change. In fact, Bournes *et al*. (1988) have reported that actin stress fibres disassemble at 32 MPa. This finding was confirmed by Crenshaw *et al*., (1996) in two other cell types: human *HeLa* fibroblasts and rat osteosarcoma cells. However, the pressure required (30-40 MPa) and the severity of the effect were variable among the cells in the culture. In particular, cells closely attached to each other, or the rare 'giant' cells, were little affected. In many cells that were rounded, the actin stress fibers disappeared but rich actin-containing microvilli appeared on the cell surface. Most of these studies were carried out in cell cultures where cells are usually flattened against the culture dish. A study in guinea pig cerebellar brain slices (Yarom, Meiri & Grossman, personal communication) has revealed that Purkinje neurons in the tissue undergo dramatic morphological change when exposed to hydrostatic pressure above 60 MPa for 10-15 min. Instead of 'rounding', the dendritic tree transformed from normal smooth cylindrical segments covered with spines (small membranous protrusions) to abnormal 'pearl necklace'- like dendrites with very low spine density. The change was irreversible when pressure exceeded 80 MPa. Cells decompressed after such exposure lost their regenerative electrical activity but not their resting potential. Although the nature of the damage in the Purkinje neurons was not determined, theoretical considerations indicate that this is the form a membrane cylinder will assume when it looses its cytoskeletal support and is unable to retract.

In skeletal muscle of fish adapted to life in the deep ocean, ΔV for actin polymerization is reduced from 100-140 $cm^3.mole^{-1}$ to 63 $cm^3.mole^{-1}$ for 3000 m dwelling fish and to only 9 $cm^3.mole^{-1}$ for 5000 m dwelling congeneric fish (Somero, 1990; 1992).

Tubulin

A single tubulin protofilament is a polymer consisting of a chain of globular heterodimers (each consists of α and β subunits). Thirteen such protofilaments in a concentric arrangement form the microtubule. This structure is dynamic; dimers may be added on one end and deleted on the other one by a complex biochemical machinery. Transport of molecules within the cell uses this system. The major component of the 'inner structure' of cilia and flagella are microtubules arranged in 9+1 sets of doublets, whereas its basal body is composed of 9 triplets. These structures are essential for the beating mechanism. A 9 triplets structure is also found in the centrioles that generate simple microtubule spindles which are formed during cell division and are responsible for chromosome polar migration. Early studies had shown that ~ 40 MPa hydrostatic pressure induces depolymerization of spindle microtubules *in vivo* in various cells (Salmon, 1975a; b), *in vitro* spindle microtubules (Salmon *et al*. 1976) or *in vitro* extracts of tubulin from rabbit brain (Salmon 1975c). However, Bournes *et al*. (1988) and recently Crenshaw *et al* (1996) have proposed that microtubules are more resistant to pressure up to 40 MPa and that it is their normal assembly in cell regions, rather than simple de-polymerisation that is impaired. ΔV for microtubule assembly was estimated to 300-400 $cm^3.mole^{-1}$ in oocytes (Salmon 1975b), but lower estimates were reported for elongation of microtubules: 26 and 50 $cm^3.mole^{-1}$ for 35° C and 15° C respectively. Since pressures

as low as 5-10 MPa were shown to be effective in slowing and modifying ciliary movement in *Paramecium* (Otter & Salmon, 1979; 1985) it was suggested that Ca^{2+} signalling to the motility mechanism is compromised. However, a recent study from the same laboratory (Crenshaw & Salmon, 1996) revealed that pressure of up to 40 MPa did not change cytosolic [Ca^{2+}] of mouse fibroblasts. Therefore, Crenshaw *et al*. (1996) have proposed that in addition to a direct effect of pressure on de-polymerisation of microtubules and certain filaments, pressure may impede some regulatory mechanism of cytoskeletal organization. A dual effect of pressure was also observed in two other ciliary systems. The beat frequency of water propelling sensory cilia in mollusc (*Hermissenda*) statocyst was gradually reduced by 30% when hydrostatically compressed to 40 MPa. At pressures higher than that, beating completely stopped (Salmon & Grossman personal communication). A similar response was observed for mucus transporting cilia in tissue cultures of frog (*Rana*) oesophageal epithelium (Waxman *et al* 1991). Ciliary arrest was observed at pressures higher than 35 MPa. In both cases, complete and fast recovery was attained upon decompression. It seems that the first decrease in activity is due to some regulatory process, whereas complete ciliary arrest is due to the direct effect of pressure on microtubules assembly.

Myosin

Myosin and actin are involved in contraction in most eukaryotic cells. The basic form of the myosin is a filamentous dimer, whose monomer is a polar molecule composed of 'tail' and 'head' regions. Under special conditions, myosin dimers aggregate through their tail regions to form bipolar 'thick filaments' of skeletal and cardiac muscle that contain hundreds of dimers. Much shorter and more labile filaments composed of only dozens of dimers, are found in smooth muscle fibres and non-muscle cells. In both types, force is generated when myosin heads interact with specific sites on the thin actin filaments, and contraction is associated with filaments sliding against each other. The process is triggered by increased cytosolic [Ca^{2+}] that induces either phosphorylation of myosin heads in smooth muscle and non-muscle cells, or enables hydrolysis of ATP by the myosin head (myosin ATPase) in skeletal and cardiac muscles. Surprisingly, myosin filaments remain stable even at pressures above 40 MPa in muscles and non-muscle types, probably due to the special tight assembly of the filament (Crenshaw *et al*., 1996). However, pressure does have a significant inotropic (positive and negative) effect on muscle contraction and its electrical activity. Since contraction is a complex process, other stages may be affected. ΔV for assembly of the actomyosin complex and the formation of high affinity cross-bridges is estimated to be approximately 100 $cm^3.mol^{-1}$, and would thus be impeded by pressure. In addition, myosin ATPase activity is inhibited by pressure, as is the response of other ATPases (for review: Hogan & Besch, 1993). Additional processes that control free cytosolic [Ca^{2+}] such as the sarcoplasmic reticulum (SR) Ca^{2+} ATPase pump and plasmalemal Na^+-Ca^{2+} exchanger activity are impeded by pressure. Consequently, at pressure cytosolic [Ca^{2+}] is expected to increase (due to the attenuated removal) and activate more rigorously muscle contraction (for review see Hogan & Besch, 1993).

There is no information about pressure effects on the other known families of motor proteins, dynein and kynesin. However, since their structure and function are similar to that of myosin, it is reasonable to assume that they will respond to high pressure exposure in a similar manner.

Intermediate filaments: Vinculin, Talin, Vimentin, Cytokeratin

These tough, stable filaments serve as mechanical support in all cell types. Their 8-10 μm diameter is intermediate: larger than the thin actin filaments and smaller than the microtubules filaments. They form rope-like strands usually of subunits consisted of triplets of coiled-coil polypeptides that vary greatly in size and amino acid sequence in different cells: these include neurofilaments in

neurons, cytokeratin filaments in epithelial cells, vimentin in fibroblast and glial cells and vinculin and talin in various types of cells. Under cellular conditions the assembly of intermediate filaments is irreversible and their disassembly requires destruction by specific proteases. However, Crenshaw *et al*. (1996) found that cytokeratin and vimentin filaments in *HeLa* cells exhibited a dramatic disruption at pressure above 20 MPa. In contrast, vimentin in rat osteosarcoma cells were not affected at pressures up to 40 MPa. Vinculin and talin filaments that are usually found in focal contact between the rat osteosarcoma cells, were absent in rounded cells at 40 MPa and were reduced in cells that remained flattened.

In summary, most cytoskeletal filaments are relatively stable at pressure. Usually pressures greater than 30 MPa and sometimes much greater are needed in order to directly disassemble the polymers. However lower pressures seem effective in impeding some regulatory mechanisms such as phosphorylation that determine cytoskeleton organization. Disruption of cytoskeletal elements occurs at pressure much higher than that reported to change ion channel function. However, if some of the cytoskeletal regulatory mechanisms are affected, reductions in filament cellular functions such as motility, contraction, adhesion, division, and morphology may be expected also at lower pressures.

Conclusion

To understand how pressure affects biological processes is an enormous scientific challenge. Unlike a chemical, pressure acts instantaneously and throughout the organism. Although this means that the confounding problems of pharmacokinetics and metabolism are not an issue, in principle every chemical reaction contributing to the continued existence of the organism is a potential target.

It appears that the biological range of pressures, from atmospheric to 100 MPa, should be divided into two. A range that can be tolerated, to various degrees, by organisms that normally live at, or close to, atmospheric pressure (up to 40 MPa) and an upper range (from 40 to 100 MPa) inhabited by organisms adapted to life at pressure. Pressures above 40 MPa can have profound effects on the molecular structure and hence function of many proteins, including cytoskeletal elements. To understand the adaptations that enable bacteria, invertebrates and some vertebrates to exist at extreme pressures will require careful analysis of the structural modifications to proteins that have occurred during the evolutionary process.

At the molecular level, pressures up to 40 MPa have little effect on enzyme function directly but may affect the phospholipid environment leading to indirect changes. It should be noted that many effects depend not only on the exposure pressure but also on the time of exposure. This suggests that these effects may play a central role in the adaptation or acclimatisation to pressure. Transporter proteins (for ionic species and amino acids) resemble enzymes in that their function is most likely to be modified by pressure affecting their environment. Cytoskeletal filaments are relatively stabile at pressure but pressure can impede some regulatory mechanisms (*e.g.* phosphorylation) that determine cytoskeleton organization. Voltage-regulated membrane bound ion channels are in the main quite resistant to pressure. Interesting exceptions are a few types of K^+ channels, which are enhanced, and N-type Ca^{2+} channels, which are depressed at pressure. Thus synapses using the latter channels may be more vulnerable to pressure exposure than others. Ionotropic receptor proteins that regulate fast chemical neurotransmission are obvious candidate targets for pressure effects. However, of the structurally related Cys-loop family of receptors only the glycine receptor, responsible for mediating inhibitory neurotransmission, is significantly affected (inhibited). The other principal family of receptors, the glutamate receptors, also show very selective pressure sensitivity. Only one of the four variants of the NMDA-sensitive glutamate

receptor is sensitive to pressure (potentiated) and the non-NMDA glutamate receptors are not affected by pressure.

Given the range of targets for modification by pressure much remains to be done before a comprehensive understanding of the effect of pressure on biological systems can be claimed**.** Indeed the existence of life at great ocean depths indicates the necessity for general adaptive mechanisms, especially of the nervous system. Finally, the prodigious diving depths achieved by the toothed whales and seals, which lie far beyond the abilities of other vertebrates, calls for special attention. It suggests that a programme to reveal the genes responsible for selected key proteins, or more ambitiously to decode their genome and compare it with ours and that of other mammals, might be the way forward.

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